Letters in Applied Microbiology 2002, **35**, 316–320

Use of *rpo*B and 16S rRNA genes to analyse bacterial diversity of a tropical soil using PCR and DGGE

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87/02: received 13 March 2002, revised 13 June 2002 and accepted 18 June 2002

R.S. PEIXOTO, H.L. DA COSTA COUTINHO, N.G. RUMJANEK, A. MACRAE AND A.S. ROSADO. 2002.

Aim: To evaluate the *rpo*B gene as a biomarker for PCR-DGGE microbial analyses using soil DNA from the Cerrado, Brazil.

Methods: DNA extraction from soil was followed by Polymerase Chain Reaction (PCR) amplification of *rpo*B and 16S rRNA genes. PCR products were compared by Denaturing Gradient Gel Electrophoresis (DGGE) to compare gene/community profiles.

Results: The *rpo*B DGGE profiles comprised fewer bands than the 16S rDNA profiles and were easier to delineate and therefore to analyse. Comparison of the community profiles revealed that the methods were complementary.

Conclusions, Significance and Impact of the Study: The gene for the beta subunit of the RNA polymerase, *rpo*B, is a single copy gene unlike 16S rDNA. Multiple copies of 16S rRNA genes in bacterial genomes complicate diversity assessments made from DGGE profiles. Using the *rpo*B gene offers a better alternative to the commonly used 16S rRNA gene for microbial community analyses based on DGGE.

INTRODUCTION

Molecular markers, such as the 16S rRNA gene, have been extensively applied to detect, identify and measure microbial diversity from environmental samples. PCR amplification of the 16S rRNA gene (rDNA), in combination with other molecular methods that generate fingerprints, such as Temperature Gradient Gel Electrophoresis (TGGE), Denaturing Gradient Gel Electrophoresis (DGGE), or Single Strand Conformation Polymorphism (SSCP), has been and is commonly used to analyse bacterial communities (Rosado *et al*. 1997; Muyzer *et al*. 1998).

Reports that some of the commonly used 'all bacteria' PCR primers, applied in DGGE community analysis, do not match the 16S rDNA targets for newly recognized groups of bacteria (Dojka *et al*. 1998) highlights the need for microbial ecologists to continually evaluate PCR primers and genes for ecological studies. The occurrence in some bacteria of multiple copies of 16S rRNA genes and sequence heterogeneity amongst these various copies complicates ecological interpretation of gel profiles (Nübel *et al*. 1996). Multiple bands on DGGE gels can result from a single species, which complicates estimations of species richness (Fogel *et al*. 1999). The possibility of mismatch primers and multiple bands from single species indicate that microbial community analyses based on 16S rDNA DGGE alone are limited.

The gene coding for the beta subunit of the RNA polymerase, *rpo*B has been proposed as an alternative biomarker for microbial community studies. This gene is described as possessing the same key attributes as 16S rDNA, in that it is common to all bacteria and is a mosaic of conserved as well as variable sequence domains (Dahllöf *et al*. 2000). Most importantly, the *rpo*B gene exists as a single copy in bacterial genomes (Mollet *et al*. 1997). Dahllöf *et al*. (2000) demonstrated that 16S rDNA heterogeneity is a typical feature of bacteria isolated from natural environments and they state that diversity indices and correlations based on the banding patterns from 16S rDNA PCR-DGGE are not suitable for monitoring and comparing changes in microbial diversity. In contrast Dahllöf *et al*. (2000) also demonstrated that PCR amplified *rpo*B genes from environmental isolates result in single bands on DGGE gels which can be correlated with single species/populations.

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This study reports on the first application of the *rpo*B gene as a biomarker for soil microbial community analysis. It compares the *rpo*B and 16S rDNA PCR-DGGE approach using DNA extracted from a Brazilian tropical soil.

MATERIALS AND METHODS

Soil sampling and DNA extraction

The soil studied was typical of the Cerrado biome, a distrophic oxisol, with a clayey texture in the sampled horizons. Samples were taken from the Cerrado Forest Reserve, Experimental Station at Embrapa Rice and Beans, Santo Antônio de Goiás, Brazil. Three samples, each consisting of a pool of three subsamples, were taken from the depths of 0–5 cm and 5–10 cm, in triplicate. Sampling was carried out on two occasions, first in June 1999 (dry season), and again in January 2000 (wet season). Soil samples were maintained at − 20 °C prior to the molecular analyses. DNA was extracted from soil using the protocol of van Elsas *et al*. (1997), which included mechanical lysis of cells, phenol and chloroform extractions, a potassium acetate precipitation and a final purification using the Wizard DNA clean up Kit (Promega, Madison, WI, USA).

PCR

The *rpo*B and 16S rDNA primers used in PCR were *rpoB* 1698F and *rpo*B 2041R (Dahllöf *et al*. 2000), 968F and 1401R (Nübel *et al*. 1996). A GC clamp (Muyzer *et al*. 1998) was added to the forward primers (F). All PCR amplifications were performed using a thermal cycler (Perkin-Elmer, Norwalk, USA). The *rpo*B PCR mixtures comprised 50 μl, containing 5 μl of Gibco *Taq* buffer 10×, 2·5 mmol l^{-1} each deoxynucleoside triphosphate, 25 pmol each primer, 20 μg of bovine serum albumin, 0·5 μl of formamide and 5 U *Taq* polymerase (Gibco*Taq*) all in sterile filtered milliQ water. The PCR program was as follows: denaturing step of 94 °C for 3 min, followed by 10 cycles of denaturing for 1 min at 94 °C, annealing for 1·5 min at 40 °C and extension for 2 min at 72 °C followed by 25 cycles of denaturing for 1 min at 94 °C, annealing for 1·5 min at 50 °C and extension for 2 min at 72 °C, and a final extension at 72 °C for 10 min.

PCR mixtures with 16S rDNA primers were prepared with 1 μl of template sample (~100 ng of DNA), 5 μl of Gibco taq buffer 10×, 2·5 mmol l^{-1} each deoxynucleoside triphosphate, 25 pmol each primer, 20 μg of bovine serum albumin, 0·5 μl of formamide and 2·5 U *Taq* polymerase (Gibco*Taq*) and sterile filtered milliQ water to a final volume of 50 μl. The PCR program was as follows: denaturing step of 94 °C for 3 min, followed by 30 cycles of denaturing for 1 min at 94 °C, annealing for 1 min at 55 °C and extension for 2 min at 72 °C, followed by a final extension at 72 °C for 10 min.

DGGE

DGGE was carried out using a Bio-Rad Dcode Universal Mutation Detection System at 75 V and 60 °C for 16 h in 0·5× TAE buffer [20 mmol l^{-1} Tris-acetate (pH 7·4), 10 mmol l^{-1} sodium acetate, 0·5 mmol l^{-1} disodium EDTA). The 6% (w/v) polyacrylamide gels were made with a denaturing gradient ranging from 45 to 70% according to the manufacturer's protocols (Bio-Rad Dcode, Richmond, VA, USA) and used with PCR products with both primer sets. After electrophoresis, gels were stained for 40 min with SYBR green I nucleic acid gel stain (Molecular Probes, Leiden, The Netherlands). The stained gels were photographed on a UV transillumination table with a Kodak digital science camera (model DC120).

Comparing community profiles

Unweighted group with mathematical averages analysis (UPGMA; Dice coefficient of similarity) using the NT-SYS software package (Exceter Software, New York, USA) was used to compare the banding patterns seen on the gels according to Gelsomino *et al*. (1999).

RESULTS

Soil DNA of sufficient purity for reproducible PCR amplification was obtained from the Cerrado soil by using the direct DNA extraction protocol of van Elsas *et al*. (1997). Eliminating the potassium acetate precipitation step of this protocol did not affect PCR amplification and significantly increased DNA yields.

A comparison of the DGGE profiles from the Cerrado soil DNA is shown in Fig. 1, where clearly the *rpo*B profiles (Fig. 1a) are different from the16S rDNA profiles (Fig. 1b) and have fewer bands. Interestingly, both the *rpo*B and the16S rDNA community profiles appear to have been little affected by season in comparison with soil depth. In general the results of the two approaches are in agreement (Fig. 2). Both methods show a 76% similarity between profiles from wet and dry seasons from the 0–5 cm soil samples. In the 5–10 cm samples the *rpo*B approach reveals 85% similarity between the seasons, whereas the 16S rDNA profiles are the same for both seasons. The ecological significance of these data is discussed elsewhere.

DISCUSSION

Direct methods for DNA extraction from soil generate the highest yields of DNA, and PCR-DGGE products with greater number of bands (Kozdrój and van Elsas 2001). In this study, adoption of standard laboratory protocols for working with soil DNA meant that clay, humic acids, and

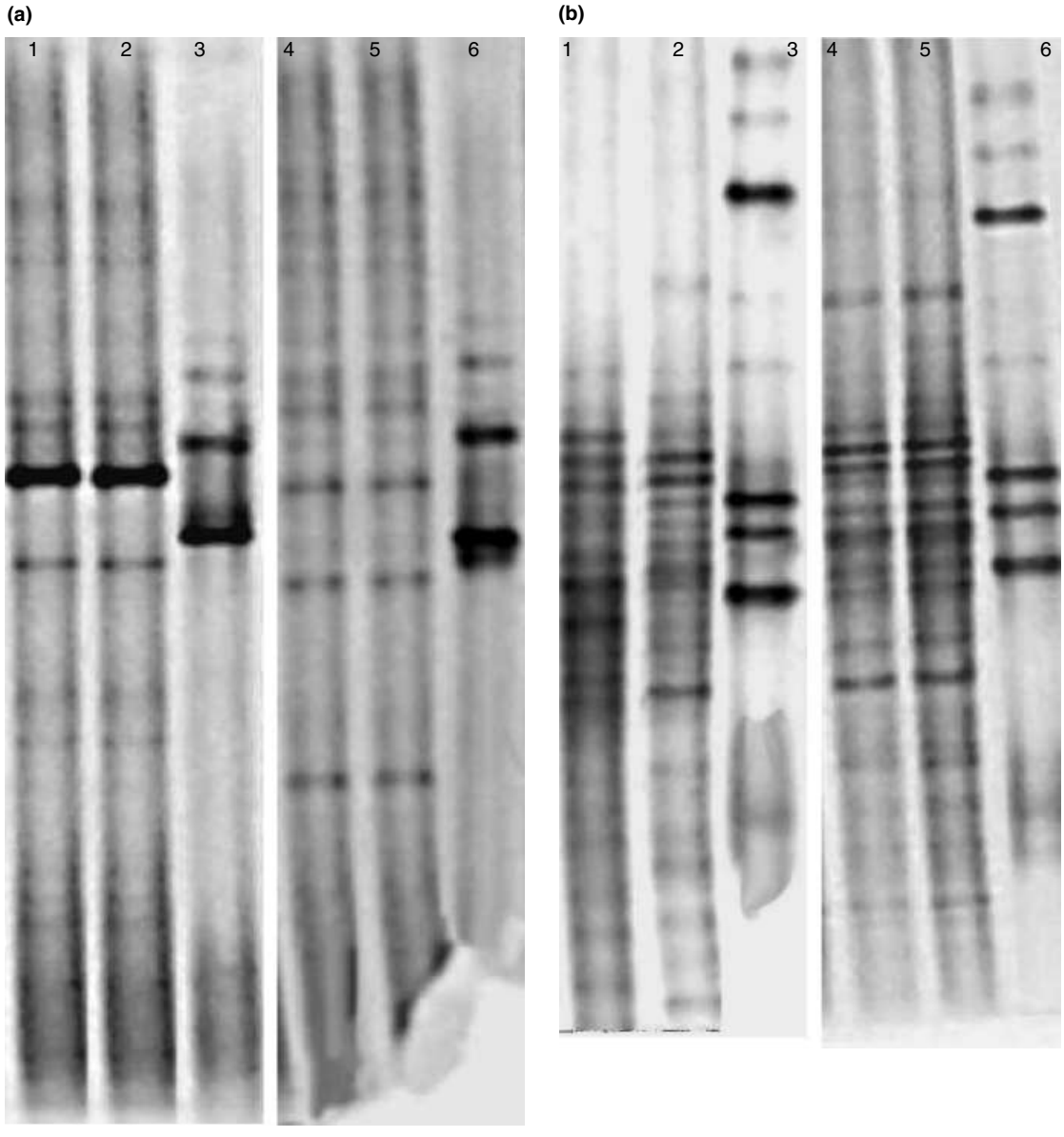

Fig. 1 *rpo*B-DGGE and 16 s rDNA-DGGE profiles from Cerrado soil Brazil. (a) *rpo*B profiles; (b) 16S rDNA profiles. In both (a) and (b), lane 1: soil samples 0–5 cm dry season; lane 2: soil samples 0–5 cm wet season; lane 4: soil samples 5–10 cm dry season; lane 5: soil samples 5–10 cm wet season. Lanes 3 and 6 show marker/control profiles (from top to bottom: *Bacillus subtilis* IS 75; *Staphylococcus aureus* MB; *Escherichia coli* HB101)

metals commonly present in soil DNA extracts (Tebbe and Vahjen 1993; Yeates *et al*. 1997) did not appear to inhibit PCR amplification of *rpo*B and 16S rRNA genes.

The PCR protocol of Dahllöf *et al*. (2000) was modified by adding BSA and formamide to the PCR mix in order to decrease *Taq* polymerase inhibition and increase PCR specificity, respectively. The number of amplification cycles was increased from 25 to 35 cycles in order to increase the amount of PCR product for DGGE analysis. Suzuki and Giovannoni (1996) have shown that an increased number of thermal cycles can result in the preferential amplification of certain target DNA. Preferential amplification is more likely in mixed-target PCR reactions, where competition between multiple targets for primer annealing results in the numerically dominant sequences being preferentially amplified (Dahllöf *et al*. 2000). If numerically dominant 16S rDNA sequences in a mix represent numerically dominant bacteria in an environment, then 16S rDNA PCR-DGGE profiles should highlight these groups of bacteria in ecological studies (Gelsomino *et al*. 1999). In this study no particular

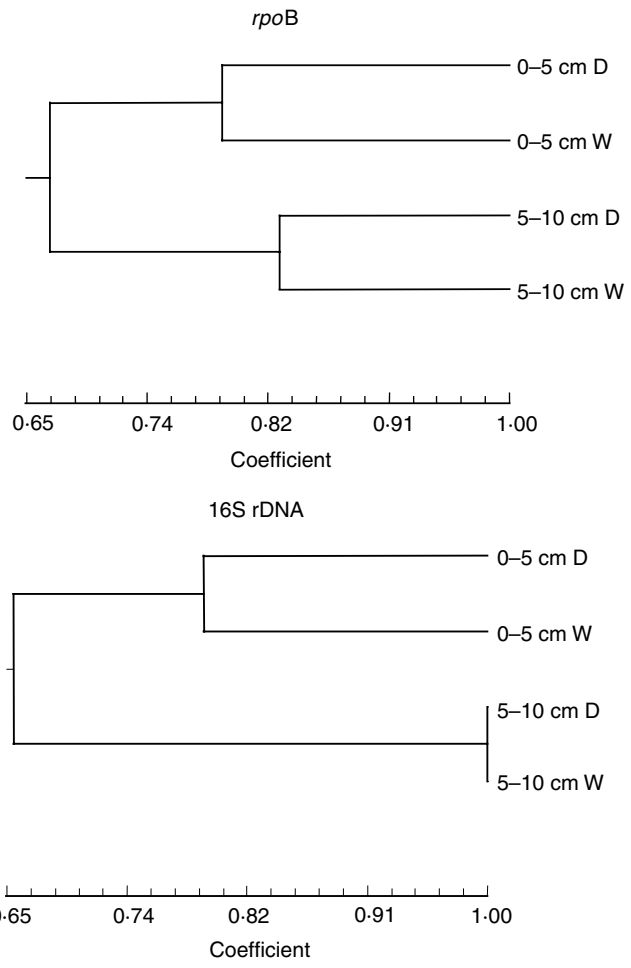

Fig. 2 Inferred similarity of bacterial communities in wet and dry seasons using *rpo*B-DGGE and 16 s rDNA-DGGE profiles from Cerrado soil, Brazil. Dendrograms were constructed from the DGGE gel photo (Fig. 1) using 1/0 clustering and the UPGMA (Dice coefficient of similarity) algorithm. Samples 0–5 cm D and 5–0 cm D represent soils sampled from 0–5 cm and 5–10 cm depth in the dry season. Samples 0–5 cm W and 5–10 cm W represent soils sampled from 0–5 cm and 5–10 cm depth in the wet season

difficulty or difference was encountered with amplification of *rpo*B and 16S rDNA genes.

Given that as many as a 1000 bacterial species may coexist in a gram of soil (Torsvik *et al*. 1990), it is clear that DGGE community profiles represent a select proportion of the total soil community. Without other confirmatory techniques such as probing back to natural environments, conclusions about microbial communities based on profiles alone are tentative. The value of DGGE and other profiling techniques to soil microbiology is understood in light of the almost unparalleled microbial diversity in soils and that the vast majority of soil bacteria have never been cultured. Much of soil microbial diversity is only known from PCR amplification and sequencing of 16S rDNA [Ribosomal Database Project (RDP) Maidak *et al*. 2001; Macrae *et al*. 2000]. PCR profiling techniques reflect a desire to sample from and monitor the majority of bacteria in natural environments not just isolated organisms. A conclusion of Gelsomino *et al*. (1999) that a 16S rDNA PCR-DGGE constitutes a reproducible strategy for measuring bacterial diversity warrants discussion. We support the assertion of Dahllöf *et al*. (2000) that reproducible multiple bands on 16S rDNA-DGGE profiles from single strains and mixes of strains demonstrate that gene heterogeneity is not an artefact of the PCR reaction. Likewise, reproducibility of banding patterns is no guarantee that the patterns are an accurate reflection of the community represented. To improve accuracy in profile analyses, single bands on gels should, as much as is possible, reflect single species in environments.

A search of all bacterial genomes available online at the GenBank Database (28/05/2002) supports the assertion of Dahllöf *et al*. (2000) that the *rpo*B gene is a single copy gene. While this remains the case, species will be represented by only one band on *rpo*B-DGGE profiles rather than potentially many bands on 16S rDNA-DGGE profiles. It follows that *rpo*B-DGGE will provide more accurate reflections of microbial communities than 16S rDNA-DGGE. In a related study Kim *et al*. (1999) have shown that comparing *rpo*B sequences can be an effective tool for species identification where 16S rRNA sequencing has failed.

In this study, the application of *rpo*B PCR-DGGE with soil DNA was straightforward and resolved differences as well as and better than 16S rDNA profiles. We have shown that the *rpoB* gene can be used as a molecular marker in using PCR and DGGE with soil DNA. We support the assertion of Dahllöf *et al*. (2000) that single copy genes are better biomarkers than heterogeneous multiple copy genes and should be chosen when possible for this type of analysis.

The adoption of good new biomarkers is important, given that no single biomarker is without limitations and multiple approaches result in a more balanced assessment of soil diversity. We will continue to compare *rpo*B genes with 16S rRNA genes from enviromental DNA and recommend that the sequencing of *rpo*B genes becomes a priority. A widely accessible database for *rpo*B sequences is now required for this promising microbial biomarker.

ACKNOWLEDGEMENTS

The authors wish to thank Lucy Seldin, Cleber Guimarães and Fabio Motta for their assistance in various forms and Embrapa, CNPq and CAPES who financially supported this work.

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